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(54) Title: THERAPEUTIC AGENTS AND AUTOIMMUNE DISEASES

(57) Abstract

There is disclosed the use, as an agent in the treatment or the prevention of an autoimmune disease, of: (i) an agent having GM-1 binding activity, other than Ctx or Etx, or the B subunits of Ctx and Etx; or (ii) an agent having an effect on GM-1 mediated intracellular signalling events, but no GM-1 binding activity. These agents may also be used in the treatment of human T cell leukaemia, in the prevention of transplant rejection or GVHD or in a vaccination method for vaccinating a mammalian subject.

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THERAPEUTIC AGENTS AND AUTOIMMUNE DISEASES

This invention relates to therapeutic agents for use in the treatment of mammalian, particularly human, autoimmune diseases. The invention also relates to

5 therapeutic agents useful in the treatment of human leukaemias of a T cell origin, as so-called "vaccine carriers", and as agents for use in the prevention of human transplantation rejection and graft versus host disease (GVHD).

10 In an article entitled "Morphologic and Functional Alterations of Mucosal T Cells by Cholera Toxin and its B subunit" by Charles O. Elson et al., The Journal of Immunology, 1995, 154; 1032-1040 it is disclosed that the cholera toxin (Ctx) and the CtxB subunit inhibit

15 CD8$^+$ and CD4$^+$ T cells.

Reference is also made to the paper entitled "Prevention of Acute Graft-Versus-Host Disease by Treatment with a Novel Immunosuppressant" by B. Yankelevich et al., The Journal of Immunology, 1995,

20 154: 3611-3617. This identifies CtxB as an agent for use in bone marrow transplantation for the prevention of acute graft-versus-host disease (GVHD).

WO 95/10301 discloses an immunological tolerance-inducing agent comprising a mucosa-binding molecule

25 linked to a specific tolerogen.

As used herein, the term "Ctx" refers to the cholera toxin and "CtxB" to the B subunit of the cholera toxin. In other texts, these may sometimes be identified as "CT" or "Ct" and "CTB" or "CtB"

30 respectively. The term "Etx" herein means the *E. coli* heat labile enterotoxin, and "EtxB" is the B subunit of Etx. In other texts, these may sometimes be identified as "LT" or "Lt" and "LTB" or "LtB" respectively.

The basis for all aspects of the present invention

35 is the finding that EtxB (the pure B-subunit of the E. coli heat labile enterotoxin) binds to GM1-ganglioside

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receptors which are found on the surfaces of mammalian
cells, and that this binding induces differential
effects on lymphocyte populations, including a specific
depletion of CD8⁺ T cells and an associated activation
5   of B cells. These effects are absent when a mutant
EtxB protein lacking GM1 binding activity is employed.

Autoimmune disease

     Autoimmunity is the term used to describe the
mechanism by which the body generates an immune
10   response to self-antigens.

     In accordance with a first aspect of the
invention, there is provided:

(i)   an agent having GM-1 binding activity, other than
      Ctx or Etx, or the B subunits of Ctx and Etx; or

15   (ii) an agent having an effect on GM-1 mediated
      intracellular signalling events, but no GM-1
      binding activity;

     for use as an agent in the treatment or the prevention
of an autoimmune disease.

20   Agents in accordance with the present invention
have been found to modulate lymphocyte populations
leading to the induction of apoptosis in CD8⁺ T cells,
the enhanced activation of CD4⁺ cells and polyclonal
activation of B cells. These events are likely to
25   shift the immune response towards induction of Th2
associated cytokines. Such responses to self or cross-
reacting antigens are understood to mediate protection
for certain autoimmune diseases.

     In a first embodiment of this first aspect of the
30   present invention, the agent is used in a method of
treating an autoimmune disease which is in progress.
In this embodiment, the agent is administered to a
patient with or without co-administration of a self or
cross-reacting antigen. Administration of the agent in
35   accordance with this embodiment of the first aspect of
the invention modulates the nature of the immune

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response towards the self-antigen away from the activation of disease-causing inflammation and hence protects against autoimmune disease.

In a second embodiment of this first aspect of the
5   present invention, the agent is used in a method for the "vaccination" of a mammalian subject against an autoimmune disease, in which the agent is co-administered with the self or cross-reacting antigenic determinant (or a combination of different self or
10   cross-reacting antigenic determinants) associated with said disease. In such a manner, the subject's immune response to the self-antigen or cross-reacting antigen is switched away from the activation of pathogenesis, which therefore protects against a future autoimmune
15   response to the self-antigen.

In this first aspect of the invention, the therapeutic agent and the self or cross-reacting antigenic determinant are, or may be, co-administered to the subject. By this we mean that the site and time
20   of administration of each of the therapeutic agent and the antigenic determinant are such that the necessary modulation of the immune system is achieved. Thus, whilst the therapeutic agent and the antigenic determinant may be administered at the same moment in
25   time and at the same site, there may be advantages in administering the therapeutic agent at a different time and to a different site from the antigenic determinant.

Whilst single doses of the therapeutic agent and the antigenic determinant may be satisfactory, multiple
30   doses are contemplated within the scope of this aspect of the invention.

In this second embodiment of the first aspect of the invention, the therapeutic agent and the antigenic determinant may be linked, for example covalently
35   linked, to form a single active agent, although separate administration, in which the therapeutic agent

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and the antigenic determinant are not so linked is
preferred because it enables separate administration of
the different moieties.

Specific autoimmune diseases which may be treated
5   in accordance with this aspect of the present invention
are the autoimmune diseases where pathology is
associated with cell-mediated immunity, such as
rheumatoid arthritis, multiple sclerosis and diabetes.

Additionally, under this first aspect of the
10   present invention, there is provided the use of Ctx,
Etx or the B subunit of Ctx or Etx, for the manufacture
of a medicament for use as an agent for the prevention
of an autoimmune disease.

Also provided is a pharmaceutical composition for
15   the treatment of a human autoimmune disease comprising
(i)   an agent having GM-1 binding activity; or
(ii) an agent having an effect on GM-1 mediated
      intracellular signalling events, but no GM-1
      binding activity;
20   and a pharmaceutically acceptable carrier or diluent
therefor.

The pharmaceutical composition of this aspect of
the invention may be formulated to be delivered by a
mucosal route, for example as a nasal spray, or
25   parenterally in which the composition is formulated in
an injectable form, for delivery by, for example, an
intravenous, intramuscular or subcutaneous route.

The pharmaceutical composition may be formulated
together with the appropriate self or cross-reacting
30   antigen. Alternatively, a kit may be provided
comprising separate compositions for each of the
therapeutic agent and the antigenic determinant.

Specific therapeutic agents which may be used in
this aspect of the invention are EtxB and CtxB or
35   mutants thereof retaining GM1 binding activity.

The agents for use in the first aspect of the

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present invention should preferably be substantially
non-toxic, although some degree of toxicity may be
tolerated in a severe therapy of this kind.

This first aspect of the invention extends to
5   cover the use of all agents having GM1 binding
activity, for use in the treatment of mammalian
autoimmune disease, as well as those agents having an
effect on GM-1 mediated intracellular signalling
events, and which therefore mimic GM-1 binding agents.

10   Thus, this first aspect of the present invention
is not limited to the use of EtxB protein as a
therapeutic agent in the treatment of a human
autoimmune disease. However, the use of the EtxB
protein (which is a pentamer of five identical
15   subunits) for such a treatment represents a preferred
embodiment of the present invention. In addition to
the wild type EtxB, this preferred aspect of the
invention also extends to mutants of EtxB which have
GM-1 binding activity as well as to other equivalent
20   proteins, such as the cholera toxin B subunit (CtxB)
and mutants thereof which have GM1 binding activity.

Other therapeutic agents for the treatment of
autoimmune disease in accordance with the first aspect
of this invention are humanised monoclonal antibodies,
25   which bind GM1. Methods known in the art for
identifying and preparing such agents are well known.

T-lymphocyte leukaemias

According to a second aspect of this invention,
there is provided:
30   (i)   an agent having GM-1 binding activity, other than
         Ctx or Etx, or the B subunits of Ctx and Etx; or
(ii) an agent having an effect on GM-1 mediated
         intracellular signalling events, but no GM-1
         binding activity;
35   for use in the treatment of human leukaemias of a T
cell origin, such as human leukaemias of a CD8 T cell

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origin.

    The agents for use in the second aspect of the present invention should preferably be substantially non-toxic, although some degree of toxicity may be

5   tolerated in a severe therapy of this kind.

    Additionally, under this second aspect of the present invention, there is provided the use of Ctx or Etx, or the B subunits of Ctx and Etx for the manufacture of a medicament for treatment of human

10 leukaemias of a T cell origin, such as human leukaemias of a CD8 T cell origin.

    Also provided is a pharmaceutical composition for the treatment of human leukaemias of a T cell origin comprising

15 (i)   an agent having GM-1 binding activity; or

(ii) an agent having an effect on GM-1 mediated intracellular signalling events, but no GM-1 binding activity;

and a pharmaceutically acceptable carrier or diluent

20 therefor.

    The pharmaceutical composition of this aspect of the invention may be formulated to be delivered by a mucosal route, for example as a nasal spray, or parenterally in which the composition is formulated in

25 an injectable form, for delivery by, for example, an intravenous, intramuscular or subcutaneous route.

    This second aspect of the invention extends to cover the use of all agents having GM1 binding activity, for use in the treatment of human leukaemias

30 of a T cell origin, as well as those agents having an effect on GM-1 mediated intracellular signalling events, and which therefore mimic GM-1 binding agents.

    Thus, this second aspect of the present invention is not limited to the use of EtxB protein as

35 therapeutic agents in the treatment of human T cell leukaemias. However, the use of the EtxB protein for

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such a treatment represents a preferred embodiment of the present invention. In addition to the wild type EtxB, this preferred aspect of the invention also extends to mutants of EtxB which have GM-1 binding
5   activity as well as to other equivalent proteins, such as the cholera toxin B subunit (CtxB) and mutants thereof which have GM1 binding activity.

        Other alternative therapeutic agents for the treatment of these diseases in accordance with this
10   aspect of the invention are humanised monoclonal antibodies, which bind GM1. Methods known in the art for identifying and preparing such agents are well known.

Transplant rejection and GVHD
15   In accordance with a third aspect of this invention, there is provided:
        (i)   an agent having GM-1 binding activity, other than
              Ctx or Etx, or the B subunits of Ctx and Etx; or
        (ii) an agent having an effect on GM-1 mediated
20   intracellular signalling events, but no GM-1
              binding activity;
        for use as a therapeutic agent for the prevention/treatment of transplant rejection or GVHD.
        Additionally, under this third aspect of the
25   present invention, there is provided the use of Ctx or Etx or the B subunit of Etx or Ctx for the manufacture of a medicament for the prevention of transplant rejection or GVHD.
        In preferred embodiments of this aspect of the
30   invention, the therapeutic agents described may be used in the prevention of solid organ transplant rejection, either allogeneic or xenogeneic. They may also be employed in the prevention of acute graft versus host disease (GVHD), for example during bone marrow
35   transplantation procedure.
        In embodiments of this aspect of the invention

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where the patient is treated prior to transplantation,
the therapeutic agent would be co-administered with
alloantigen or xenoantigen. In embodiments in which
the patient is treated after transplantation, the
5   therapeutic agent is employed without co-administration
of antigen.

In the embodiment of this aspect of the invention,
where the therapeutic agent and allo- or xeno-antigenic
determinant are co-administered to the subject, we mean
10   that the site and time of administration of each of the
therapeutic agent and the antigenic determinant are
such that the necessary modulation of the immune system
is achieved. Thus, whilst the therapeutic agent and
the antigenic determinant may be administered at the
15   same moment in time and at the same site, there may be
advantages in administering the therapeutic agent at a
different time and to a different site from the
antigenic determinant. Furthermore, the therapeutic
agent and the antigenic determinant may be covalently
20   linked to form a single active agent, although separate
administration, in which the therapeutic agent and the
antigenic determinant are not so linked is preferred
because it enables separate administration of the
different moieties.

25   Whilst single doses of the therapeutic agent and
the antigenic determinant may be satisfactory, multiple
doses are contemplated within the scope of this aspect
of the invention.

In this aspect of the invention, where the agent
30   is being used in the prevention of GVHD, the agent
would normally be applied direct to the cells, for
example bone marrow cells, to be transplanted.

The agent is preferably substantially non-toxic,
although some degree of toxicity may be tolerated in
35   severe therapies of this kind.

Also provided is a pharmaceutical composition for

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use in the treatment of transplant rejection,
comprising
(i)   an agent having GM-1 binding activity; or
(ii) an agent having an effect on GM-1 mediated
5   intracellular signalling events, but no GM-1
          binding activity;
and a pharmaceutically acceptable carrier or diluent
therefor.

The pharmaceutical composition of this aspect of
10 the invention may be formulated to be delivered by a
mucosal route, for example as a nasal spray, or
parenterally in which the composition is formulated in
an injectable form, for delivery by, for example, an
intravenous, intramuscular or subcutaneous route.

15   The pharmaceutical composition may be formulated
together with the appropriate allo- or xeno-antigeneic
determinant. Alternatively, a kit may be provided
comprising separate compositions for each of the
therapeutic agent and the antigenic determinant.

20   This third aspect of the invention extends to
cover the use of all agents having GM1 binding
activity, for use in the prevention/treatment of
transplant rejection or GVHD, as well as those agents
having an effect on GM-1 mediated intracellular
25 signalling events, and which therefore mimic GM-1
binding agents.

Thus, this third aspect of the invention is not
limited to the use of EtxB protein as a therapeutic
agent in the treatment of a transplant rejection.
30 However, the use of the EtxB protein (which is a
pentamer of five identical subunits) for such a
treatment represents a preferred embodiment of the
present invention. In addition to the wild type EtxB,
this preferred aspect of the invention also extends to
35 mutants of EtxB which have GM-1 binding activity as
well as to other equivalent proteins, such as the

cholera toxin B subunit (CtxB) and mutants thereof which have GM1 binding activity.

Other alternative therapeutic agents for the treatment of transplant rejection in accordance with
5   the invention are humanised monoclonal antibodies, which bind GM1. Methods known in the art for identifying and preparing such agents are well known.

Vaccination

CtxB and EtxB have already been suggested as so-
10 called "vaccine carriers". It has now been discovered that the basis for this effect, in part, is the ability of EtxB to modulate lymphocyte populations (as discussed above) by binding to the GM-1 receptor.

Thus, in accordance with a fourth aspect of the
15 present invention, there is provided:
    (i)   an agent having GM-1 binding activity, other than Etx or Ctx or the B subunits of Etx or Ctx; or
    (ii) an agent having an effect on GM-1 mediated intracellular signalling events, but no GM-1
20   binding activity;
    for use in the vaccination of a mammalian subject.

The agent is capable of modulating the immune response when delivered together with an unrelated foreign antigenic determinant. Where the agent is
25 delivered parenterally, such immunomodulation is in terms of the immune response being "directed" in a particular desired direction. Where the agent is delivered mucosally with an unrelated antigen, as a so-called "mucosal adjuvant", the agent is capable of
30 facilitating a mucosal immune response to the unrelated antigen. The antigen and agent may be delivered together as separate moieties, or may be linked together, for example by a covalent linkage.

The agent is preferably non-toxic. In addition,
35 where the agent is to be delivered mucosally through the gastrointestinal mucosa, it should be able to

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remain stable during transit through the
gastrointestinal tract; for example, it should be
resistant to proteolytic degradation, stable at acid pH
and resistant to the detergent effects of bile.

5   Also provided is a pharmaceutical composition for
use in the vaccination of a mammalian subject,
comprising
(i)   an agent having GM-1 binding activity; or
(ii)   an agent having an effect on GM-1 mediated
10   intracellular signalling events, but no GM-1
            binding activity;
and a pharmaceutically acceptable carrier or diluent
therefor.

           The pharmaceutical composition of this aspect of
15 the invention may be formulated to be delivered by a
mucosal route, for example as a nasal spray, or
parenterally in which the composition is formulated in
an injectable form, for delivery by, for example, an
intravenous, intramuscular or subcutaneous route.

20   The pharmaceutical composition may be formulated
together with the appropriate antigenic determinant.
Alternatively, a kit may be provided comprising
separate compositions for each of the therapeutic agent
and the antigenic determinant.

25   This fourth aspect of the invention extends to
cover the use of all agents having GM1 binding
activity, as immunomodulators, as well as those agents
having an effect on GM-1 mediated intracellular
signalling events, and which therefore mimic GM-1
30 binding agents.

           Thus, this fourth aspect of the invention is not
limited to the use of EtxB protein as an
immunomodulator. However, the use of the EtxB protein
(which is a pentamer of five identical subunits) in
35 such a way represents one embodiment of the present
invention. In addition to the wild type EtxB, this

preferred aspect of the invention also extends to
mutants of EtxB which have GM-1 binding activity as
well as to other equivalent proteins, such as the
cholera toxin B subunit (CtxB) and mutants thereof
5   which have GM1 binding activity.

Other alternative therapeutic agents for use as an
immunomodulator in accordance with this aspect of the
invention are humanised monoclonal antibodies, which
bind GM1. Methods known in the art for identifying and
10   preparing such agents are well known.

When the therapeutic agent of the invention is a
protein, such as the EtxB subunit or the CtxB subunit,
it may be produced, for use in all aspects of this
invention, by a method in which the gene or genes
15   coding for the specific polypeptide chain (or chains)
from which the protein is formed, is inserted into a
suitable vector and then used to transfect a suitable
host. For example, the gene coding for the polypeptide
chain from which EtxB assemble may be inserted into,
20   for example, plasmid pMMB68, which is then used to
transfect host cells, such as <u>Vibrio</u> sp.60. The
protein is purified and isolated in a manner known <u>per</u>
<u>se</u>. Mutant genes expressing active mutant EtxB protein
may then be produced by known methods from the wild
25   type gene.

As previously stated, agents having GM-1 binding
activity, such as specifically designed humanised
monoclonal antibodies, may be designed and produced as
outlined above, by methods which are known in the art.
30   In all aspects of the invention, the agent having
GM1 binding activity may also be capable of cross-
linking GM1 receptors. EtxB is one such agent which is
capable of cross-linking GM1 receptors by virtue of its
pentameric form.
35   The invention will now be illustrated by reference
to the accompanying drawings and the following

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examples.

<u>BRIEF DESCRIPTION OF THE DRAWINGS</u>

    Figure 1 represents an analysis of physico-
chemical properties of EtxB and a mutant form of EtxB,
5   (EtxB(G33D));

    Figure 2 illustrates that receptor binding by EtxB
is essential for its potent immunogenicity *in vivo*;

    Figure 3 illustrates the kinetics of lymphocyte
proliferation following injection of mice with EtxB;

10   Figure 4 illustrates that EtxB causes increased
activation of B cells;

    Figure 5 illustrates that EtxB causes increased
activation of CD4$^+$ T cells and depletion of CD8$^+$ cells.

    Figure 6 shows the selective depletion of OVA-
15 responsive CD8$^+$ T cells by EtxB;

    Figure 7 shows that receptor binding by EtxB
induces alterations in lymphocyte nuclear morphology
characteristic of cells undergoing apoptosis;

    Figure 8 shows EtxB receptor-mediated apoptosis of
20 CD8$^+$ T cells as measured by cell cycle analysis;

    Figures 9a and 9b show the results of experiments
conducted to show that GM-1 binding by EtxB inhibits
the development of collagen induced arthritis in an
animal model;

25   Figure 10 shows the results of an experiment
conducted to illustrate that EtxB but not EtxB(G33D)
induces apoptosis in a population of normal human
peripheral blood mononuclear cells; and

    Figure 11 shows the results of an experiment which
30 shows that cross-linking of GM1 leads to apoptosis in a
proportion of murine CTLL cells.

<u>DETAILED DESCRIPTION OF THE DRAWINGS</u>

<u>Figure 1</u>

Analysis of physico-chemical properties of EtxB and
35 EtxB (G33D).

    (A) SDS-PAGE analysis of EtxB or EtxB (G33D): 5µg

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of each protein were analysed under reducing conditions
in the presence of ß-mercaptoethanol with or without
prior heating. Lane 1, wild type EtxB, unheated. Lane
2, EtxB (G33D), unheated. Lane 3; wild type EtxB,
5   heated at 95°C. Lane 4, EtxB (G33D), heated at 95°C.
Molecular weight standards (BioRad) are shown on the
left-hand side of the panel.

(B) Determination of apparent molecular mass of
EtxB and EtxB (G33D) by gel filtration chromatography:
10   standard curve (circles) was generated using, from top
to bottom: bovine serum albumin (66 kDa), hen egg
albumin (45 kDa), bovine erythrocyte carbonic anhydrase
(29 kDa) and horse heart cytochrome c (12.4 kDa); EtxB
and EtxB (G33D) eluted with apparent molecular masses
15   of 36kDa and 38kDa respectively; Ve-elution volume of
the protein, Vo-void volume of the gel filtration
column.

(C) ELISA for comparative binding of EtxB and EtxB
(G33D) to ganglioside GM1: plates were coated with GM1,
20   blocked and incubated with 1μg/ml of either EtxB or
EtxB (G33D) diluted serially (3 fold) from 1μg/ml.

Figure 2

**Receptor binding by EtxB is essential for its potent
immunogenicity in vivo.**

25   BALB/c mice (4 in each group) were either injected
s.c. with EtxB or EtxB (G33D) in PBS or given the
proteins orally in bicarbonate buffer. Sera were
analysed 10 days following two s.c. injections (A) or 1
week following 3 oral doses (B), and gut secretions
30   were analysed 1 week following 3 oral doses (C). No
reaction was detected in samples from control mice (not
shown). Results are expressed as mean IgG antibody
titre in serum, while IgA in gut secretion is expressed
as 'specific activity' as described below.

35   **Figure 3**

Kinetics of lymphocyte proliferation

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Mice were injected i.p. with 30 μg of EtxB (G33D) in complete Freund's adjuvant. MLNs were isolated 10 days later and cells were incubated in the absence of antigen (open square) or in the presence of 80 μg/ml

5   EtxB (filled triangles), EtxB (G33D) (open triangles) or their disassembled monomeric forms of EtxB (filled circles) and EtxB (G33D) (open circles) generated by heating at 95°C. The last 6 hour on each sampling day cells were pulsed with 1μCi of (^{3}H) Thd. Data

10 represents mean cpm and SEM of triplicate wells.

Figure 4

EtxB causes increased activation of B cells.

Mice were immunized with EtxB (G33D) in CFA. Cells were isolated from MLN 10 days later and

15 incubated in the presence of 80μg/ml of either EtxB or EtxB (G33D) or a mixture of 40μg/ml of each protein. Cells were labelled with biotinylated anti-CD25 (7D4) and Phycoerythrin (PE) anti-B220 (Ra3-6D2). Streptavidin FITC was used as a secondary antibody

20 conjugate. Controls for the antibodies were also included (not shown). Dual flow cytometric analysis was performed on day 4 of proliferation.

Figure 5

EtxB causes increased activation of CD4+T cells and

25 **depletion of CD8*T cells.**

The immunization procedure, cell isolation and the *in vitro* challenge are as described in the legend of Fig. 4. To detect CD25, biotinylated anti-CD25 (7D4) and Streptavidin FITC were used. To detect CD4 and

30 CD8, FITC labelled anti-CD4 (RNRM4-5) and FITC labelled anti-CD8α (53-6.7) were used. Appropriate controls for the antibodies were included (not shown).

Figure 6

Selective depletion of OVA-responsive CD8* T cells by

35 **EtxB**

Cultures of cells from MLN taken from OVA-primed

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mice were established for 5 days, in the absence of
antigen or in the presence of OVA + EtxB, OVA +
EtxB(G33D) or OVA alone at 100 µg OVA and 40 µg/ml each
of EtxB or EtxB(G33D) or 100 µg OVA alone. Cells were
5   labelled with the following rat antibodies: FITC-anti-
CD4 or FITC-anti-CD8α and both with biotin-anti-CD25
(IL-2Rα) followed by Streptavidin-phycoerythrin. Non-
stained cells or cells stained with the second antibody
alone were also included as controls. Cells were
10 analysed by FACS (Becton Dickinson). The higher
increase in the proportion of total cells which are
CD25+ in cultures containing EtxB compared with other
treatments is due to the presence of higher proportion
of B cells expressing this marker (not shown). The
15 scale of fluorescence intensity is log.

Figure 7

Receptor binding by EtxB induces alterations in
lymphocyte nuclear morphology characteristic of cells
undergoing apoptosis.

20   MLNC comprising >90% CD3⁺ T cells and depleted of
macrophages were incubated for 18 h with either 80
µg/ml EtxB or with 80 µg/ml EtxB(G33D) and stained with
acridine orange. Cells were examined under conventional
or confocal fluorescence microscopy (Leica TCS 4D). A
25 representative microscopic field (x 540) for each
treatment is shown [EtxB, left hand panel; EtxB (G33D),
right hand panel]. Cells which were incubated in the
absence of antigen gave similar results to those
treated with EtxB(G33D) (not shown).

30 Figure 8

EtxB receptor-mediated apoptosis of CD8⁺ T cells as
measured by cell cycle analysis.

        The proportion of CD4⁺ and CD8⁺ SPLTC in the sub-
G₀/G₁ stage of the cell cycle was determined by flow
35 cytometric analysis of the DNA content following
staining with propidium iodide. SPLTC were isolated

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from the spleen by negative selection as described
above. The cells were treated for 18 h with: (a) no
antigen, (b) 80 μg/ml EtxB(G33D) or (c) 80 μg/ml EtxB
and then stained with FITC-rat anti-CD4 or FITC-rat
5   anti-CD8α. The cells were subsequently stained with
propidium iodide. The proportion of cells co-stained
with propidium iodide was determined by gating on cells
stained with either anti-CD4 or anti-CD8 antibodies.
This experiment has been carried out on cells, results
10   of which are also reported in Figure 7 and Table 3.

Examples

Example 1

This example illustrates the requirement for GM-1
15   binding to induce differential effects on lymphocyte
populations.

Materials and Methods

Generation of a receptor-binding mutant of EtxB

A Gly-33 to Asp substitution was introduced into
20   the receptor binding site of human EtxB using plasmid
pTRH29, a derivative of the phagemid vector pBluescript
IIKS+, that contains the genes for the A- and B-
subunits of Etx (Yu, J., Webb, H. & Hirst, T.R. (1992),
Molec. Microbiol. 6, 1949-1958). Mutagenesis was
25   performed with an *in vitro* oligonucleotide-directed
mutagenesis kit (Amersham International) using
single-stranded pTRH29 as a template and a synthetic
oligonucleotide (5'-TCTCTTTTATCTGCCATCG-3') (from the
Microanalytical Facility, IAPGR, Cambridge Research
30   Station, UK) as the mutagenic primer. The correct Gly
to Asp substitution was confirmed by dideoxy sequencing
using Sequenase II (United States Biochemical Corp.)
and the resultant plasmid was designated pTRH56. The
mutant *etxB* gene from pTRH56 was excised, using *Eco*RI
35   and *Spe*I restriction enzymes, and inserted into pMMB68
(Sandkvist, M., Hirst, T.R. & Bagdasarian, M. (1987) J.

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Bacteriol. 169, 4570-4576) to yield a broad host range
expression vector, pTRH64 expressing EtxB(G33D).

Antigens

    Wild-type EtxB and EtxB(G33D) were purified from
5   culture supernatants of *Vibrio* sp.60 (pMMB68) and
Vibrio sp.60 (pTRH64), respectively, using a
modification of the method reported by Amin and Hirst
(Amin, T., & Hirst, T.R. (1994) Prot. Express. and
Purif. 5, 198-204). Briefly, proteins were purified by
10   diafiltration and hydrophobic interaction
chromatography and concentrated by anion-exchange
chromatography. The protein solutions were desalted on
a PD10 column (Pharmacia, UK) equilibrated with
phosphate buffered saline (PBS; 10mM sodium phosphate,
15   150 mM NaCl, pH7.4) and stored at -30°C.

    The purity of EtxB and EtxB(G33D) were confirmed
by SDS polyacrylamide gel electrophoresis. The
molecular mass of the individual monomers were
confirmed by laser desorption mass spectrometry
20   (Protein Science Facility, University of Kent).

    Apparent molecular masses of EtxB and EtxB(G33D)
were determined by gel filtration chromatography using
a SMART system (Pharmacia). Proteins were eluted from a
Superdex 75 PC 3.2/30 column in PBS, pH7.5.

25   Irreversible denaturation of B subunit pentamers,
for use in lymphocyte proliferation assays (see below),
was achieved by heating the proteins at 95°C for 5 min.

Animals, sample collection and immunization protocols

    BALB/c mice (H-2^d; high responder to EtxB) of 7-12
30   weeks of age were purchased from Charles River
Laboratories and maintained at the University of Kent
animal house. Antibody responses to EtxB or EtxB(G33D)
were measured after s.c. injection of mice with 30µg of
protein in PBS, followed by boosting 10 days later.
35   Another group of mice were given the same protein dose
orally in sodium bicarbonate (50µg/ml) on 3 occasions,

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and at one week intervals. Control mice were given
PBS. Blood was collected 10 days following the last
s.c. injection or one week following the last oral
feeding. Gut secretions from live mice were isolated

5   in a protease inhibitor solution as previously
described (Elson, C.O., Ealding, W. & Lefkowitz, J.
(1984) J Immunol. Meth. 67, 101- 108), one week
following the last feeding. Samples were then
sonicated and clarified by centrifugation (13,226 x g,

10   10 min, at 4°C).

For the proliferative assays, mice were injected
i.p. with 30µg of EtxB or EtxB(G33D) in complete
Freund's adjuvant (CFA) and the mesenteric lymph nodes
isolated 10 days later. Control unimmunized mice were

15   also included and their lymph nodes isolated in a
similar manner.

Enzyme Linked Immunosorbent Assays (ELISAs)

Binding of EtxB or EtxB(G33D) to GM1 was examined
by a GM1-ELISA (Amin, T., & Hirst, T.R. (1994) Prot.

20   Express. and Purif. 5, 198-204).

Sera and gut secretions were examined for the
presence of anti-B subunit IgG and IgA antibodies by
ELISA's in which samples were applied to microtitre
plates (Immulon I, Dynateck, USA) coated with 5µg/ml of

25   either EtxB or EtxB (G33D) in PBS. Anti-B subunits IgA
antibodies in gut secretion supernatants were
extrapolated from a standard curve made by coating 2
rows of wells on each plate with 1µg/ml rabbit
anti-mouse IgA (α chain specific; Zymed Lab, USA) in

30   PBS followed by addition of 1µg/ml of mouse myeloma IgA
(MOPC 315, Sigma, USA). To measure total IgA, wells
were coated with rabbit anti-mouse IgA followed by
addition of gut secretion supernatants. All samples
were serially diluted. Goat anti-mouse IgG (Fc

35   fragment specific; Jackson Lab., USA) or goat
anti-mouse IgA (a chain specific; Sigma) peroxidase

conjugate were diluted and added to all wells. The
anti-B subunit IgG titer, giving an $A_{450nm} \geq 0.2$, was
determined. The IgA anti-B subunit response for each
of EtxB and EtxB (G33D) in gut secretions was

5   calculated as "IgA specific activity" [mean IgA anti-B
subunit ($\mu g/ml$)/total IgA ($\mu g/ml$)].

An ELISA method for measuring cytokine levels of
IL-2, IL-4, IL-5, IL- 10 and IFN-γ was used, as
described previously (Harper, H.M., PhD thesis,

10 Univeristy of Bristol (1995)). Briefly, microtiter
plates were coated with rat antibodies to mouse IL-2,
IL-4, IL-5, IL-10 and IFN-γ. Plates were blocked with
2% (w/v) bovine serum albumin. Supernatants from
culture medium were added to wells and diluted down.

15 One row on each plate for each cytokine contained a
standard amount of recombinant cytokines. Plates were
then incubated with $0.5\mu g/ml$ of biotinylated
anti-cytokine monoclonal antibodies followed by
addition of avidine-peroxidase and

20 3,3',5,5'-Tetramethylbenzidene (TMB) substrate and read
at A_{405nm}.

Lymphocyte proliferation assay

Mice were sacrificed by cervical dislocation,
mesenteric lymph nodes were excised aseptically and

25 minced through a stainless steel mesh into Hank's
balanced salt solution (HBSS) (Flow Laboratories,
Irvine, Renfrewshire, UK.). Cells were washed by
centrifugation (500 x g, 10 min, 4°C) in HBSS and
resuspended in modified Eagle's medium (Flow) to which

30 20mM Hepes (Flow), 100 IU Penicillin, $100\mu g/ml$
Streptomycin, 4mM L-glutamine (Flow) and
2-mercaptoethanol had been added (complete medium).
Fresh autologous normal mouse serum from unimmunized
mice was added to a final concentration of 0.5% (v/v).

35 Cultures contained 2×10^6 viable cells/ml in either 2
ml volumes in 24-well plates or 8 ml volumes in 25 cm^3

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flasks (Nunc A/S, Roskide, Denmark) and were
established in the presence and absence of antigens as
indicated in the figures legend. Cultures were
incubated at 37°C in a humidified atmosphere of 5% CO_2

5   and 95% air for 6 days. At desired timepoints, 0.1 ml
samples were removed from the cultures and transferred
to 96 well U-bottomed plates (Nunc) and pulsed with
$1\mu Ci$/well of $[^3H]$-Thd (Amersham, U.K) for 6 h before
harvesting (Mach III harvesting 96 Tomtec, Orange,

10 Conn. USA) and counting by standard liquid
scintillation 1450 Micro ß plus, LKB-Wallac, Turku,
Finland). Similarly, 0.5 ml of supernatant was sampled
from cultures for cytokine analysis. Cells were
pelleted and the supernatants stored at - 68°C until

15 analysed.
Phenotypic analysis of cultured cells
    Cultured cells harvested on day 4 of culture were
washed and viable cells recovered at the interface of a
HBSS/18% metrizamide (Nyegaard and Co., Oslo, Norway)

20 gradient following centrifugation at 500 xg for 15 min
at 20°C. Cells were washed twice and resuspended in
HBSS containing 0.2 % sodium azide (Sigma) and 10%
normal rat serum. The following rat antibodies
(Pharmingen, San Diego, USA) were used: fluorescein

25 isothiocyanate (FITC) labelled anti-CD4 (RNRM4-5), FITC
labelled anti-CD8 (53-6.7), biotin-labelled anti-CD25
(7D4) and Phycoerythrin (PE) labelled anti-B220
(RA3-6D2). Additionally, for the biotin-labelled
antibodies Streptavidin-PE or Streptavidin-FITC

30 (Serotech, UK) were used. All antibodies were diluted
in HBSS containing azide and used at predetermined
concentrations. $200\mu l$ of 2 x 10^6 cells and $200\mu l$ of
each the antibodies were mixed and incubated on ice for
30 min. When Streptavidin -PE or FITC secondary

35 antibodies were required cells were incubated with
these antibodies for additional 30 min. Appropriate

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controls for FITC and PE antibodies were also included.
Cells were washed with HBSS and then analysed by 2 flow
cytometery (Becton Dickinson).

<u>Results</u>

5   **Generation and characterization of a receptor binding
     mutant of EtxB**

        A Gly to Asp substitution was introduced into the
     B subunit of *E. coli* heat-labile enterotoxin by
     oligonulceotide-directed mutagenesis of EtxB, in order
10   to generate a mutant B subunit defective in receptor
     recognition. The mutant protein, designated
     EtxB(G33D), and wild type EtxB were purified to
     homogeneity (see Materials and Methods). The molecular
     mass of purified EtxB and EtxB(G33D) were determined by
15   laser desorption mass spectrometry. Masses were within
     20 Da of the theoretical masses of 11702 and 11760 Da
     for monomeric EtxB and EtxB (G33D), respectively. When
     analysed by SDS-PAGE without prior heating, both
     wild-type EtxB and EtxB(G33D) migrated as discrete
20   stable oligomers, with apparent molecular weights of 42
     kDa and 56 kDa (Fig.1A, lane 1 and lane 2,
     respectively). The observed electrophoretic mobility
     and SDS-stability of EtxB is a characteristic property
     of the B subunit pentamer (see Sandkvist, M., Hirst,
25   T.R. & Bagdasarian, M. (1987) J. Bacteriol. 169, 4570-
     4576). The slower electrophoretic mobility of
     oligomeric EtxB(G33D) is not due to a difference in the
     number of constituent B subunit monomers, since both
     pentameric EtxB and EtxB(G33D) exhibited similar
30   retention times when analysed by high resolution gel
     filtration chromatography. Thus, the discrepancy in
     the electrophoretic mobility of the EtxB(G33D) oligomer
     with respect to wild-type EtxB, is likely to be due to
     the introduced negatively charged Asp residue causing a
35   reduction in SDS binding and a subsequent slower
     migration.

EtxB and EtxB(G33D) were also compared for their
stability in low pH buffers, resistance to 1.0 mg/ml of
either trypsin or proteinase K, and relative reactivity
towards a panel of anti-B subunit monoclonal and
5   polyclonal antibodies. In each of these tests
EtxB(G33D) exhibited identical properties to wild-type
EtxB. It is therefore concluded that a Gly to Asp
substitution at residue 33 in EtxB does not alter the
oligomeric configuration, SDS, pH or protease
10   stability, or antibody reactivity compared with
wild-type EtxB.

The ability of EtxB(G33D) to bind to its receptor
GM1, was evaluated using a GM1-ELISA (Fig. 1C). This
showed a highly significant reduction in the ability of
15   the mutant to bind GM1 compared with the wild type
protein (> 99% reduction in the A_{450nm} reading).
Furthermore, in contrast to wild type EtxB, EtxB(G33D)
failed to bind to CHO cells when examined by
immunofluorescence. It is concluded that EtxB(G33D) is
20   defective in its capacity to bind GM1 ganglioside, in
vitro and in situ.

**The potent immunogenicity of EtxB in vivo is dependent
on receptor binding**

The importance of receptor binding in the
25   immunogenicity of EtxB was evaluated in mice following
either oral delivery or s.c. injection of EtxB or
EtxB(G33D) in PBS. Oral delivery of EtxB resulted in
detection of a high IgG antibody titer in serum and IgA
antibody activity in gut secretions (Fig. 2). In
30   contrast, a similar regime of oral immunization with
EtxB(G33D) failed to generate any detectable antibody
activity. EtxB(G33D) did induce a serum antibody
response following s.c. injection, although the
response was considerably lower in comparison to the
35   antibody response to wild type EtxB, with > 160 fold
reduction in the mean antibody titer, 1050 versus

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171000, respectively. It is concluded that receptor
binding by EtxB is essential for its potent
immunogenicity *in vivo*.

Receptor binding does not influence the extent of
5   **lymphocyte proliferation in the presence of EtxB or**
EtxB(G33D)

The effect of EtxB or EtxB(G33D) on lymphocyte
proliferation *in vitro* was examined. Lymphocytes were
isolated from the popliteal and mesenteric lymph nodes
10   (MLN) of mice immunized with either EtxB or EtxB(G33D)
and stimulated *in vitro* with either protein, or with a
heat-denatured preparation of EtxB or EtxB(G33D). The
proliferative response of lymphocytes derived from the
popliteal or MLN was similar. In each case,
15   proliferation to each of the protein preparations
increased with increasing B subunit concentration. A
representative set of data from an experiment using MLN
is shown in Table 1. The magnitude of the response to
wild type and mutant pentamers was comparable as was
20   that in the presence of heat-denatured wild type and
mutant monomers. Figure 3 shows the kinetics of the
proliferative responses obtained in the presence of 80
µg/ml of each of the protein preparations. Reactivity
was dependent on the presence of antigen, and followed
25   a similar pattern in the presence of each protein.
Reactivity was evident on day 3 of culture with
incorporation of [³H]-Thd reaching a peak on day 4 and
waining thereafter. The minor differences in the
timing of peak responses apparent in Figure 3 were not
30   observed in repeat experiments, showing that the
anamnestic characteristics of the responses to the EtxB
and EtxB(G33D) are comparable. It is concluded that
the level of stimulation in the presence of the native
proteins is not likely to be influenced by receptor
35   binding or the introduced mutation.

Toxin receptor binding causes immunomodulation of B

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cells and T cell subsets

To examine if receptor binding by EtxB exerts any
effect on the populations of lymphoid cells *in vitro*,
lymphocytes were isolated from the MLN of mice primed

5   i.p. with EtxB(G33D) and then stimulated with either
EtxB or EtxB(G33D) or a mixture of both. Additionally,
a parallel experiment using MLN-derived lymphocytes
from mice injected with EtxB was undertaken and
resulted in essentially identical findings to those

10   obtained from EtxB(G33D) primed mice.

(i) EtxB causes increased activation of B cells

The effect of EtxB on B cells were examined by
expression of the activation marker CD25 (IL-2Rα) in
association with the B cell marker B220 (CD45R). As

15   shown in Fig.4 the number of B cells in cultures
stimulated with EtxB was 62.9% of total cells, of which
a high proportion (28.4%) expressed the cell activation
marker CD25. In contrast, the proportion of B cells
after stimulation in the presence of EtxB(G33D) was

20   less than half of that of the wild type (22.26%) and
fewer were activated (5.6%). To establish whether the
effects exerted by EtxB were dominant, cells were
incubated in the presence of an equimolar concentration
of EtxB and EtxB(G33D). The flow cytometric data was

25   similar to that obtained following stimulation in the
presence of wild type EtxB alone (with 60.6% B cells,
of which 26% were activated). It is concluded that the
receptor binding property of EtxB mediates an increased
activation of B cells *in vitro*.

30   **(ii) EtxB causes increased activation of CD4⁺ T cells
and complete depletion of CD8⁺ T cells.**

To examine the influence of B subunit receptor
binding on T cells, lymphocytes were labelled with
antibodies to CD4 or to CD8 in association with

35   antibodies to CD25 (Fig.5). Additionally, some cells
were separately labelled with antibodies to the CD3

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marker (not shown). The proportion of T cells expressing the CD4 marker when stimulated in the presence of EtxB was 36.7%, of which a high proportion (32.7%) were activated. In contrast, no detectable CD8·

5   T cells were present in the culture containing EtxB.

By comparison, both CD4· and CD8· T cells were present in the culture stimulated in the presence of EtxB(G33D). Such cultures contained a large proportion of CD4· T cells (66.6%), but only 12% of these were

10   activated. The proportion of CD8· T cells detected in the presence of EtxB(G33D) was 11.7% of the total number of cells, but very few of these were activated which is indicated by the absence of the CD25 marker. Additionally, in the presence of a mixture consisting

15   of an equimolar concentration of EtxB and EtxB(G33D) the pattern of responding cells was similar to that in the presence of wild type EtxB alone; with 41.68% CD4· T cells (of which 28.6% were CD25+) and no detectable CD8· T cells (Fig.5). In all these analyses, the proportion

20   of cells staining with CD3 was approximately equal to the sum of those expressing CD4 and CD8 markers. These data demonstrate that the increase in activation of B and CD4· T cells and the selective depletion of CD8· T cells are mediated by toxin receptor occupancy.

25   **Production of cytokines**

To assess whether the effect of EtxB on lymphocyte populations could be dependent on a change in cytokine production, cell cultures were incubated with either EtxB or EtxB(G33D) and supernatants removed on days 2,

30   3, 4, 5 and 6 for analysis. The results from samples collected on day 5 are shown in Table 2 when the maximum concentration of cytokines was detected. Both IFN-γ and IL-2 were detected in the supernatants from cultures stimulated in the presence of EtxB or

35   EtxB(G33D), although the relative levels of these cytokines varied. The medium from cells incubated with

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wild type EtxB, contained a 3-fold higher concentration
of IL-2 and a 1.5 fold lower level of IFN-γ compared
with supernatants from cultures stimulated in the
presence of EtxB(G33D). Despite the finding that other

5   proliferating T cell cultures responding to other
antigens yielded high levels of IL-4, IL-5 and IL-10
none of these cytokines were detected in cultures
stimulated with EtxB or EtxB(G33D). The increase in
the level of IL-2 and decrease in the level of IFN-γ

10 following stimulation with EtxB, compared with
EtxB(G33D), most likely reflects the activation status
of B and CD4⁺ T cells. Nonetheless, the results
indicate that the profound effect of wild type EtxB on
the

15 CD8⁺ T cell population is unlikely to be mediated by a
major shift in the cytokine profile, as a consequence
of receptor occupancy.

Discussion

These investigations show that the introduction of
20 a single point mutation (G33D) in the receptor binding
site of EtxB caused a significant loss in the ability
to bind GM1. Importantly, the mutant EtxB(G33D),
exhibited identical physico-chemical properties to the
wild type EtxB with respect to conformation, as

25 revealed by gel chromatography, stability in SDS, acid
and proteases. When the specific antibody responses
were measured following immunization with either EtxB
or EtxB(G33D), dramatic differences were noted.
Subcutaneous injection with EtxB(G33D) in mice resulted

30 in a highly significant drop in the antibody titer
compared with wild type (ca >160 folds) while no
antibody response was detected following oral
administration. It is possible that these differences
result from the disruption of a dominant epitope

35 involved either in the recognition of the molecule by
antibody, or the stimulation of effective T cell help

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for antibody production. However, it is noteworthy
that the Gly to Asp substitution had no effect on the
recognition of the B subunit by a panel of specific
polyclonal and monoclonal antibodies. Further, the
5 proliferative responses obtained when EtxB or
EtxB(G33D) were added to cultures were comparable
regardless of which of the proteins were used for in
vivo priming; demonstrating that the T cell reactivity
was not specific to either molecule. It is therefore
10 concluded that receptor binding by EtxB is essential
for its potent immunogenicity in vivo.

The importance of receptor binding in the potent
immunogenicity of EtxB may be explained in a number of
ways. Firstly, binding of the B subunit of Etx and Ctx
15 to GM1 may increase the efficiency of uptake of these
proteins, raising the local protein concentration
available to the immune system. Other classes of
proteins which are able to bind mucosal surfaces are
found to be effective immunogens (De Aizpura, H.J. &
20 Russell-Jones, G.J. (1988) J. Exp. Med. 167, 440- 451).
The observed differences in the immunogenicity of EtxB
and its mutant following oral administration may indeed
be due to efficient uptake of EtxB from the lumen of
the gut. However, the dramatic differences noted after
25 parenteral immunization (where antigen is delivered
locally at high concentration) are suggestive of other
effects. For example, binding of EtxB to GM1 may
affect the efficiency of antigen presenting cell
activity. Such binding could cause activation of class
30 II-bearing cells, particularly with respect to their
expression of essential co-stimulatory molecules, such
as B7, which is associated with their acquiring
enhanced antigen presenting activity (Jenkins, M.K. &
Johnson, J.G. (1993) Curr. Opin. Immunol. 5, 361-367).
35 Alteratively, receptor binding may have direct effects
on sub-populations of lymphocytes. A number of

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observations from this study provide strong evidence that this is indeed the case.

The *in vitro* studies demonstrated that EtxB was able to induce the proliferation of primed lymph node cells. This property was not dependent on receptor binding, since responses with similar anamnestic characteristics were obtained using either wild type EtxB, EtxB(G33D) or heat-denatured monomeric forms of these proteins which cannot bind GM1. These observations are interesting in themselves since it has been widely reported that commercial preparations of Ctx and CtxB or purified recombinant CtxB are strongly inhibitory of lymphocyte proliferation *in vitro*. The apparent discrepancy may have arisen from the fact that previous experiments had been conducted on purified lymphocytes and had largely used mitogen stimulated lymphocyte cultures (which are not clonally restricted responses), where a different mechanism may be involved. Consistent with this was our observation that the proliferation of Con A-stimulated lymphocytes was indeed inhibited by EtxB. However, the analyses of cell populations in cultures of primed lymph node cells stimulated with either EtxB or EtxB(G33D) revealed important differences with respect to B cells as well as CD4 and CD8-bearing T-cells.

B cells were detected after 4 days of culture in the presence of either EtxB or EtxB(G33D). However, by comparison with EtxB(G33D), the relative proportion of B cells present in cultures with EtxB was increased by approximately 100%. This increase was associated with the expression of CD25 on a very high proportion of the B cells. In the experiment shown, the responding lymphocytes were primed with EtxB(G33D) *in vivo*. Similar experiments with cells from EtxB immunized mice revealed comparable results. Thus, irrespective of any *in vivo* effects associated with receptor binding,

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cultures in the presence of EtxB contained a larger
proportion of B cells compared with those stimulated
with EtxB(G33D). These effects on B cells also appear
not to be dependent, at least in part, on regulation by
5   T cells, *in vitro*, as the results do not suggest a
major shift in the profile of the detected cytokines.
Therefore, *in vitro*, receptor binding by EtxB appears
to be associated with a direct effect on B cells,
resulting in proportional expansion of this population
10 as well as their activation. It is also noteworthy
that CtxB has been shown to increase expression of MHC
class II on virgin B cells, a property which was not
exhibited by a GM1 binding mutant CtxB (G33E) (Francis,
M.L., Ryan, J., Jobling, M.G., Holmes R.K., Moss, J, &
15 Mond J.J. (1992) J. Immmunol. 148, 1999-2005). The
results in these experiments suggest the presence of
direct mitogenic effects by EtxB on antigen-primed B
cells and demonstrate that such effects are mediated by
receptor binding.

20   In addition to the effects of EtxB on B cells in
culture, flow cytometric analyses reveal that this
toxoid caused the complete depletion of any detectable
CD8$^+$ cells. Once again, this effect was shown to be
dependent on receptor binding, since this population of
25 T cells were not depleted in cultures containing
EtxB(G33D). Further, complete depletion of CD8$^+$ cells
in cultures containing EtxB was observed, from mice
immunized with wild type EtxB. There are three
possible mechanisms by which such an effect may be
30 mediated. 1) It is known that binding of Ctx or CtxB to
GM1 on rat MLN cells induces patch and cap formation
(Craig S.W. and Cuatrecasas P., (1975) Proc. Natl.
Acad. Sci. USA, Vol.72, pages 3844-3848). It is
possible that in this process EtxB-GM1 complexes and
35 other molecules, including CD8, are internalized. Such
a process would prevent flow cytometric detection of

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these cells using CD8 as a marker, and may result in
their death due to the associated loss of the surface
TCR complex. Although the latter may account for the
absence of CD8⁻ T cells in the culture, others found no
5   loss of the TCR complex from the surface of human
Jarkat T cell line when CtxB was used (Imboden, J.B.,
Shoback, D. M., Pattison, G, & Stobo, J.D. (1986) Proc.
Natl. Acad. Sci. USA 83, 5673-5677). Absence of
effects as a result of capping is supported by the
10   finding that CD3 and CD4 markers were not affected. 2)
An alterative mechanism would involve effects exerted
by cytokines in culture. In this study, both IL-2 and
IFN-γ were detected. The results, however do not
suggest a major shift in the cytokine profile which
15   would explain such a dramatic effect on CD8⁻ T cells. 3)
Absence of CD8⁻ T cells may be due to active induction
of apoptosis. Death of lymphocytes by apoptosis may
involve capping as described above, or could be
mediated in the absence of capping by effects on the
20   signalling events in the cell. Activation-induced
programmed death is dependent on Ca²⁺ and involves
phosphatases and kinases. Binding of CtxB to
lymphocytes has been shown to inhibit protein kinase
C-dependent proliferation and induced a pronounced
25   increase in intra-cellular Ca²⁺, events which were not
associated with an increase in CAMP level. The ability
of EtxB to deplete CD8⁻, but not CD4⁺ T cells could be
due to differential effects of signals associated with
the CD4/CD8-TCR complex, resulting from crosslinking
30   GM1 on the surface of these subset of lymphocytes.
This could be as a result of differential binding of
the toxoid on the membrane as reported for CtxB or
alternatively to the differential signalling mechanisms
in CD4⁻ and CD8⁻ T cells.
35   In the complete absence of detectable CD8⁻ T cells,
EtxB increased the proportion of CD4⁻ T cells which were

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activated, by comparison with the receptor binding
mutant. The essential requirement for CD4⁺ T cells in
response to Ctx has been demonstrated *in vivo*. The
reason for the increased activation of this T cell

5   subset is, however, unclear. It is noteworthy that
CtxB has been shown to stimulate DNA synthesis and cell
division in quiescent non-transformed mouse 3T3 cells.
A selective mitogenic effect on CD4⁺ T cells was also
found in the presence of plant lectins which bind to

10 Galß-1-3-3GalNAc, the same component that EtxB binds to
in GM1. The possibility can not be ruled out that EtxB
mediates a GM1-binding dependent direct effect on CD4⁺ T
cells, causing their activation. However, it is also
possible that the increased activation of CD4⁺ T cells

15 in cultures containing EtxB is a consequence of those
changes to the B cell and CD8⁺ T cells populations
described. B cell activation is known to be associated
with an enhancement of their competency as antigen
presenting cells for CD4⁺ T cells. Further, CD8⁺ T

20 cells are widely associated with a regulatory role in
immune reactivity both *in vivo* and *in vitro*. Their
removal from T cell proliferative cultures has been
associated with prolonged and enhanced levels of CD4⁺ T
cell division.

25   Taken together, the potent immunogenicity of EtxB
in vivo, as shown in this study, can be suggested to
occur as a result of its ability to increase activation
of B cells exerted by growth regulating effects
following binding to GM1. Activation of CD4⁺ T cells

30 and the ability of EtxB to increase production of IL-2
in culture *in vitro* may provide the necessary signal
for further expansion of B cell clones. Depletion of
CD8⁺ T cells by EtxB *in vitro* in this study may also
provide another mechanism of immunopotentiation *in vivo*

35 following systemic or oral delivery, particularly in
the light of the involvement of this subset of cells in

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suppression of the immune response and in oral
tolerance. In this regard, both Ctx and Etx have been
shown to abrogate oral tolerance to cofed soluble
proteins and other studies implicated Ctx and CtxB
5   depletion effects on intra-epithelial lymphocytes in
the gut or in the dome of Peyer's patch to explain this
mechanism. CtxB-inhibitory effects on CD8⁺T cells *in
vitro* has also been shown to prevent graft versus host
reaction.

10   In conclusion, it has been demonstrated that the
presence of potent immunomodulatory effects by EtxB on
the antibody response *in vivo*, and on populations of
lymphocytes *in vitro*. Furthermore, it has been
demonstrated that these effects are mediated by
15   receptor binding. Our findings are also pertinent to
an understanding of the ability of Etx and Ctx to act
as potent adjuvants and as potential protein carriers
for other antigens and suggest that such properties
rely on the capacity of these toxoids to bind
20   ganglioside receptors on the surface of lymphoid cells.

Example 2

This example illustrates that the effects on CD8
cells are irrespective of antigen recognition and are
mediated by apoptosis.

25   Recombinant preparations of EtxB and EtxB(G33D)
were prepared as in Example 1. Both proteins were well
characterised with respect to binding to GM1, binding
to a panel of monoclonal and polyclonal antibodies and
various other physico-chemical properties. Ovalbumin
30   (OVA) was purchased from Sigma (Poole, UK). Mesenteric
lymph nodes (MLN) were isolated from BALB/c mice [high
responder strain to EtxB (Nashar, T.O. and Hirst, T.R.
1995. Immunoregulatory role of H-2 and intra-H-2
alleles on antibody responses to recombinant
35   preparations of B-subunits of *Escherichia coli* heat-
labile enterotoxin (rEtxB) and cholera toxin (rCtxB).

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Vaccine 13:803.)] 8-10 weeks old. Mice were injected
i.p. with 200μg of OVA (Sigma) emulsified in incomplete
Freund's adjuvant (Sigma). MLN were removed 10 days
after injection, minced through a stainless steel mesh

5   into HBSS (Flow, Irvine, UK). The recovered cells were
washed in HBSS by centrifugation (500 g, 10 min, 4°C)
and resuspended in modified Eagle's medium (Flow)
containing 20 mM HEPES, 100 IU penicillin, 100 μg/ml
streptomycin, 4 mM L-glutamine and 5 x 10^{-5} M 2-

10   mercaptoethanol (complete medium) to which 0.5% (v/v)
of fresh autologous mouse serum was added. Cultures
contained $2x10^6$ viable cells/ml in 2 ml volumes in 24-
well plates (Nunc, Roskide, Denmark) and were
established in the presence of 100 μg/ml OVA (dialysed

15   extensively in complete medium), either alone or with
40 μg/ml of EtxB or EtxB(G33D). Cultures were incubated
at 37°C in 5% CO_2 and 95% air for 5 days. At desired
time points, 0.1 ml samples were removed from the
cultures and transferred to 96 well U-bottomed plates

20   (Nunc) and pulsed with 1 μCi/well of [^{3}H]thymidine
(Amersham, UK) for 6 h before harvesting (Mach III
harvesting 96; Tomtec, Orange, CT) and counting by
standard liquid scintillation (1450 Micro b plus; LKB-
Wallac, Turku, Finland). For flow cytometric analysis

25   (Becton Dickinson, Erenbodegem-Aalst, Belgium) of T
cells, cells were stained with the following rat
antibodies (PharMingen, Cambridge, UK): FITC labelled
anti-CD4 (RNRM4-5) or FITC- anti-CD8α (53-6.7) and with
biotin-labelled anti-CD25 (IL-2Rα) (7D4) followed by

30   Streptavidin-phycoerythrin. Additionally, for the
biotin-labelled antibodies FITC-labelled Streptavidin
was used. FACS analysis of recovered cells was
performed on the peak day of proliferation (day 4), as
determined by [^{3}H]thymidine incorporation.

35   For apoptosis assays, fresh MLN cells (MLNC) and
splenic T cells (SPLTC) were isolated from BALB/c mice,

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8-10 weeks old. MLNC comprising >90% CD3⁺ T cells, as
determined by flow cytometric analysis, were incubated
for 2 h in petri dishes (Costar, Cambridge, MA) in
complete medium containing 10% FCS, at 37° C in 5% CO_2
5　and 95% air to remove adherent cells. The non-adherent
fraction was subsequently pipetted off, pelleted and
washed twice in HBSS before use. SPLTC were purified by
negative selection using glass beads coated with normal
mouse serum followed by rabbit anti-mouse γ-globulins
10　as described (Wigzell, H. 1976. Specific affinity
fractionation of lymphocytes using glass or plastic
bead columns. *Scand. J. Immunol.* 5:(suppl.5) 23.). The
selected population of T cells were >90% CD3⁺ as
determined by flow cytometric analysis.

15　　　CD4⁺ and CD8⁺ T cells were separated as follows:
non-adherent MLNC were labelled with rat phycoerythrin-
anti-mouse CD4 (4708-02) or FITC-anti-mouse CD8α (53-
6.7) (PharMingen) and were then incubated with MACS
colloidal super-paramagnetic microbeads conjugated with
20　goat anti-rat IgG (H + L) F(ab')₂ (PharMingen),
according to the manufacturer's instructions. These
were applied to mini-MACS columns (Miltenyi Biotec,
Bergisch Gladbach, Germany) in order to separate both
positively (>99% pure) and negatively (>90% pure)
25　selected populations of CD4 and CD8+ T cells, as
determined by flow cytometric analysis.

　　Two methods were used for quantification of
apoptosis: i) staining DNA with acridine orange to
examine nuclear morphology and, ii) cell cycle analysis
30　following staining DNA with propidium iodide and with
either anti-CD4 or anti-CD8 antibodies. Cultures of
2×10^6/ml MLNC, SPLTC and fractionated MLNC were
established in complete medium containing 10% FCS, in
the absence or presence of 80 μg/ml of either EtxB or
35　EtxB(G33D) and examined from 4 to 18 h. Following
incubation, cells were pelleted, washed with HBSS and

stained with 5 μg/ml acridine orange (Sigma).
Thymocytes were isolated and treated in the absence or
in the presence of 10^{-7}M dexamethasone and used as a
positive control for cells undergoing apoptosis.

5   Nuclear morphological changes in lymphocytes were
examined by conventional or confocal fluorescence
microscopy (Leica TCS 4D). The proportion of $CD4^*$ and
$CD8^*$ SPLTC in the sub-G_0/G_1 stage of the cell cycle was
determined by flow cytometric analysis of the DNA

10   content following staining with propidium iodide as
described (O'Connor, P.M., Jackman, J., Jondle, D.,
Bhatia, K., Magrath, I. and Kohn, K.W. 1993. Role of
p53 tumor suppressor gene in cell cycle arrest and
radiosensitivity of Burkitt's lymphoma cell lines.

15   *Cancer.Res.* 53:4776.). Cells isolated from 18 h
cultures of SPLTC incubated alone or with 40 μg/ml EtxB
or EtxB(G33D) were stained with FITC rat anti-CD4 or
FITC-anti-CD8α. Stained cells were adjusted to 1×10^6/ml
in cold HBSS containing 20mM HEPES and 0.5 mM EDTA and

20   were fixed with cold ethanol added dropwise. Then, 50
μg/ml propidium iodide and 40 μg/ml ribonuclease A
(DNase free) were added, and the cells incubated for 1
h at room temperature. The relative intensity of DNA
staining with propidium iodide in CD4 and $CD8^*$ T cells

25   was determined by gating on cells co-stained with each
mAB.

        In Example 1, the observation that $CD8^*$ T cells are
completely depleted from cultures of lymph node cells
proliferating in response to EtxB suggested that EtxB

30   exerts a polyclonal effect on this T cell subset. To
investigate whether such effects are dependent on the
activation of EtxB responsive cells, cultures were
established from OVA-primed mice and stimulated with
OVA alone or with OVA plus either EtxB or the mutant

35   EtxB(G33D). Similar peak levels of proliferation (day 4
of culture in each case) were achieved in the presence

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of OVA alone, OVA plus EtxB or OVA plus EtxB(G33D)
(9734 ± 347, 12,031 ± 135 and 9305 ± 290 c.p.m.
respectively). However, there was a dramatic difference
in the distribution of T cell subsets in these cultures

5   after 4 days (Figure 6). All cultures contained CD4⁺ T
cells of which similar proportions co-expressed the
activation marker CD25. However, CD8⁺ T cells were
undetectable in cultures incubated with OVA plus EtxB,
but were clearly present (although not activated as

10 assessed by CD25 expression) in cultures with OVA plus
EtxB(G33D) or OVA alone. This establishes that EtxB
induces depletion of CD8⁺ T cells responding to an
antigen other than EtxB. Moreover, the absence of such
a response to EtxB(G33D) indicates that depletion is

15 triggered following toxoid receptor interaction. It was
also noted that the presence of wild-type EtxB caused a
significant increase in the proportion of B cells of
which a large number were CD25⁺ (not shown) as had
previously been found for EtxB responsive cultures

20 (Example 1). It is therefore concluded that receptor
occupancy by EtxB exerts profound immunomodulatory
effects on lymphocytes irrespective of their antigen
specificity.

          The possibility that CD8+ T cells undergo

25 apoptosis when cultured in the presence of EtxB was
investigated. MLNC or purified SPLTC, from unprimed
mice, were incubated with EtxB or EtxB(G33D) and
changes in cell nuclear morphology after staining with
acridine orange were recorded over a period of 4 to 18

30 h (Table 3 and Figure 7). Cell morphological changes
were characterized by the presence of condensation of
chromatin resulting in the lobular appearence of the
nucleus (Figure 7). Other cell features such as
blebbing of the plasma membrane and the presence of

35 apoptotic bodies were also observed. These
morphological changes occurred in approximately one

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third of each of the cell preparations treated with
EtxB, whereas a much lower incidence was observed in
cells cultured with EtxB(G33D) or without exogenous
antigen (Table 3). Since CD8⁻ T cells accounted for
5   ~35-40% of the MLNC and SPLTC preparations, depletion
of these cells could account for the observed
apoptosis. To establish if this was the case,
populations of purified CD8 and CD4⁻ T cells were
cultured for 18 h in the presence of antigens (Table
10   3). Similar percentages of morphological changes were
induced in negatively selected populations of CD4⁻ T
cells (containing >90% CD4-bearing cells) on treatment
with either EtxB, EtxB(G33D) or no antigen, indicating
that binding of EtxB to its receptor does not trigger
15   apoptosis in this T cell subset. In contrast, >70% of
the negatively selected CD8⁻ T cells (>90% pure)
exhibited morphological changes when cultured with
wild-type EtxB; while incubation with either no antigen
or EtxB(G33D) caused changes in only 11-19% of this T
20   cell population, respectively. Further, the presence
of low numbers of contaminating cells in the purified
populations used (~10% in each case) could not account
for the observed effects since more highly purified
populations containing >99% of CD8 or CD4⁻ T-cells
25   (isolated by positive selection) responded to EtxB in a
similar manner (60% were apoptotic in the presence of
EtxB, compared with 7% for both no antigen and
EtxB(G33D) treatments) (Table 3). Apoptosis was
detected in 40% and 98% of thymocytes after 18 h
30   incubation in the absence or in the presence of
dexamethasone respectively.

       To demonstrate that the morphological changes
observed in our cultures were consistent with the
induction of apoptosis, the appearance of subdiploid
35   DNA in cultures of SPLTC treated for 18 h with EtxB was
evaluated. Cells were subjected to flow cytomeric

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analysis after co-staining with propidium iodide and
either anti-CD8 or anti-CD4 antibodies (Figure 8).
Approximately 48 % of the CD8⁺ T cells from cultures
incubated with EtxB fell below the diploid G_0/G_1 peak of

5   propidium iodide staining, indicating that they were
undergoing apoptosis (O'Connor, P.M. et al, *supra*). A
small proportion of cells expressing CD4, in cultures
with EtxB, also exhibited sub-G_0/G_1 levels of DNA (~11%;
which may result from the death of such a high

10   proportion of CD8⁺ T cells). In contrast, the majority
of CD4 or CD8⁺ T cells cultured without antigen or in
the presence of EtxB(G33D) were in G_0/G_1 phase of the
cell cycle, with <5% exhibiting apoptosis. We conclude
that the observed nuclear morphological changes, and

15   the presence of sub-G_0/G_1 levels of DNA, in a
substantial proportion of CD8⁺ T cells treated with EtxB
demonstrate a selective apoptosis triggered by the
cholera-like enterotoxoid. The failure of the receptor
binding mutant, EtxB(G33D), to induce similar effects

20   demonstrates that the induction of CD8⁺ T cell apoptosis
is linked to its ability to bind to GM1 ganglioside.

Example 3

     Groups of 8 male DBA/1 mice were either
unchallenged (group A) or were each injected with $100\mu g$

25   of bovine collagen in CFA on day 0 by intra-dermal
(i.d.) injection into the flank. Collagen injected
mice were either left unprotected (group B; positive
control) or attempts were made to prevent disease
development by the administration i.d. at an adjacent

30   site to collagen challenge of; $100\mu g$ of EtxB in IFA on
day 0 (group C), $100\mu g$ of EtxB in IFA on day 14 (group
D), or $100\mu g$ EtxB(G33D) in IFA on day 0 (group E). All
animals, except those in group A, received a boosting
dose of collagen in IFA i.d. on day 21, and disease

35   severity was assessed on day 45 by measuring hind limb
ankle thickness (experiment A) or scoring each hind

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limb digit for swelling (scale 0-3 where 0 = normal,
and 3 = maximal swelling; experiment B).

    The results obtained are illustrated in Figures 9a
and 9b. These show that EtxB, but not EtxB(G33D),
5   dramatically protects mice from the development of
collagen-induced arthritis.

Example 4a

    Two separate human buffy coat samples (obtained
from normal human blood donors) were used as a source
10 of mononuclear cells. Cells were isolated over Ficoll-
paque and washed extensively before culture in the
absence of antigen or with 80μg/ml of either EtxB or
EtxB(G33D) as indicated. Prior to culture the cell
populations comprised 24% CD8+, 27% CD4+ and 27% CD8+,
15 22.9% CD4+ for each sample respectively. After culture
for 18 hours the appearance of apoptotic cells was
assessed in samples of cells stained with acridine
orange (as detailed under Example 2). The results
obtained are shown in Figure 10; these illustrate that
20 EtxB but not EtxB(G33D) induces apoptosis in a
population of normal human peripheral blood mononuclear
cells.

Example 4b

    The murine T cell line, CTLL-2, was cultured to
25 confluence and then the cells washed before being
reseeded at 1 x 10^6 cells/ml in the absence of antigen
or with 80μg/ml of either EtxB or EtxB(G33D) as
indicated. After 18 hours samples were removed and the
percentage of cells showing signs of apoptosis was
30 assessed using acridine orange (detailed under Example
2). The results obtained are illustrated in Figure 11.
They show that cross-linking of GM1 leads to apoptosis
in a proportion of murine CTLL cells.

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Table 1 - Lymphocyte proliferation in the presence of
EtxB or EtxB (G33D)

Dose µg/ml	EtxB	EtxB(G33D)	EtxB*	EtxB(G33D)*
0	117.9 (7.9)	146.8 (3.5)	124.5 (14.6)	116.1 (6.35)
5	4928 (98.7)	2860 (3.8)	2424 (88.3)	1431.5 (37.5)
10	6978 (30.6)	3681 (4.6)	2518 (21.6)	4231 (96.4)
20	7084 (100)	6912 (47.3)	4394 (42.1)	5075 (24.8)
40	8844 (26)	8586 (143.7)	7431 (45.3)	4368.5 (118.9)
80	10246 (30.7)	12510 (121.8)	7986 (210.3)	7276 (369.5)
160	11311 (247)	13525 (352.7)	-----	-----

Mice were injected i.p. with 30µg of EtxB (G33D) in
complete Freund's adjuvant (CFA). Mesenteric lymph
nodes were isolated 10 days later. Cells were isolated
and incubated for 4 days in the presence of EtxB, EtxB
(G33D) or disassembled monomeric forms of these
proteins (*), generated by heating at 95°C.
Proliferation was determined by addition of 1µCi of (^{3}H)
dThd for the last 6 hours on day 4. Data represents
mean cpm and SEM of triplicate wells. Cells isolated
from unimmunized mice gave <1500 cpm (dose 160µg/ml).

Table 2 - Cytokine analysis in the presence of EtxB or
EtxB (G33D)

Protein	IL-2 (pg/ml)	IFN-γ (pg/ml)
EtxB	318	2700
EtxB (G33D)	67	4068

Mice were injected with EtxB (G33D) in CFA and
mesenteric lymph nodes cells were isolated 10 days
later. Cells were then incubated in vitro with either

EtxB or EtxB(G33D) and samples of supernatants analysed for cytokine content on day 5 of cellular proliferation.

5 Table 3 - EtxB-receptor mediated apoptosis in fractionated lymphocyes.

Cells	Time (h)	No antigen	EtxB(G33D)	EtxB
MLNC	4	0^a (8)	2 (0)	1 (3)
	18	8 (10)	5 (18)	29 (35)
SPLTC	4	3 (7)	2 (6)	3 (5)
	18	17 (5)	16.5 (12)	31 (32)
Negative selection				
CD4	18	5 (37)	6 (31)	9 (35)
CD8	18	18 (11)	19 (15)	76 (73)
Positive selection				
CD4	18	6	4	6
CD8	18	7	7	60

20 Nuclear morphological changes in fractionated CD4 and CD8[*] T cells after 4 or 18 h incubation in the absence of antigen, or with 80 μg/ml of EtxB or EtxB(G33D) were examined by fluorescence microscopy following staining with acridine orange. Whole MLN were depleted of

25 adherant cells. SPLTC were isolated by negative selection in glass beads column coated with mouse γ-globulins and rabbit anti-mouse as a secondary antibody. Fractionated SPLTC were obtained following labelling with rat phycoerythrin-anti-mouse CD4 or

30 FITC-anti-mouse CD8α which were then incubated with MACS colloidal super-paramagnetic microbeads conjugated with goat anti-rat IgG (H + L) F(ab')$_2$. These were separated using mini-MACS columns to obtain both the positively (>99% pure) and negatively (>90% pure)

35 selected fractions of CD4 and CD8[*] T cells. Nuclear morphological changes were examined from 4 to 18 h in a random sample of 200 cells per treatment as described in the legend to Fig. 7. Maximum percentage of

apoptotic cells occurred after 18 h. The data in
brackets when indicated represent results from another
separate experiment. Data for MLN and SPLTC are
representative of a total of four experiments.

5   [a]Percentage apoptotic cells

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CLAIMS:

1.   For use as an agent in the treatment or the
prevention of an autoimmune disease:

(i)   an agent having GM-1 binding activity, other
5   than Ctx or Etx, or the B subunits of Ctx and
               Etx; or

(ii)   an agent having an effect on GM-1 mediated
               intracellular signalling events, but no GM-1
               binding activity.

10   2.   The use of Ctx, Etx or the B subunit of Ctx
or Etx, for the manufacture of a medicament for use as
an agent for the treatment or the prevention of an
autoimmune disease.

3.   A method for treating an autoimmune disease
15 in which an effective amount of an agent selected from:

(i)   an agent having GM-1 binding activity; or

(ii)   an agent having an effect on GM-1 mediated
               intracellular signalling events, but no GM-1
               binding activity;

20 is administered to a mammalian subject.

4.   A method according to claim 3, wherein said
agent is co-administered with a self or cross-reacting
antigen.

5.   A pharmaceutical composition for the
25 treatment of a mammalian autoimmune disease comprising:

(i)   an agent having GM-1 binding activity; or

(ii)   an agent having an effect on GM-1 mediated
               intracellular signalling events, but no GM-1
               binding activity;

30 and a pharmaceutically acceptable carrier or diluent
therefor.

6.   A pharmaceutical composition according to
claim 5, further comprising self- or cross-reacting
antigenic determinant.

35   7.   A kit comprising a pharmaceutical composition
in accordance with claim 5 and separate therefrom a

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pharmaceutical composition comprising self- or cross-
reacting antigenic determinant.

8.   For use in the treatment of human leukaemias
of a T cell origin:

5 (i) an agent having GM-1 binding activity, other than
        Ctx or Etx, or the B subunits of Ctx and Etx; or

(ii) an agent having an effect on GM-1 mediated
        intracellular signalling events, but no GM-1
        binding activity.

10   9.   The use of Ctx or Etx, or the B subunits of
Ctx and Etx for the manufacture of a medicament for
treatment of human leukaemias of a T cell origin.

10.   A method for treating a human leukaemia of a
T cell origin in which an effective amount of an agent

15 selected from:

        (i)   an agent having GM-1 binding activity; or
        (ii) an agent having an effect on GM-1 mediated
              intracellular signalling events, but no GM-1
              binding activity;

20 is administered to a mammalian subject.

11.   A pharmaceutical composition for the
treatment of human leukaemias of a T cell origin
comprising:

        (i)   an agent having GM-1 binding activity; or
25   (ii) an agent having an effect on GM-1 mediated
              intracellular signalling events, but no GM-1
              binding activity;

and a pharmaceutically acceptable carrier or diluent
therefor.

30   12.   For use as a therapeutic agent for the
prevention/treatment of transplant rejection or GVHD:

        (i)   an agent having GM-1 binding activity, other
              than Ctx or Etx, or the B subunits of Ctx and
              Etx; or

35   (ii) an agent having an effect on GM-1 mediated
              intracellular signalling events, but no GM-1

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binding activity.

13.   The use of Ctx or Etx or the B subunit of Etx or Ctx for the manufacture of a medicament for the prevention of transplant rejection or GVHD.

5   14.   A method for the prevention or treatment of transplant rejection or GVHD in which an effective amount of an agent selected from:

(i)   an agent having GM-1 binding activity; or

(ii) an agent having an effect on GM-1 mediated
10   intracellular signalling events, but no GM-1
              binding activity;

is administered to a mammalian subject.

15.   For use in the vaccination of a mammalian subject:

15   (i)   an agent having GM-1 binding activity, other
              than Etx or Ctx or the B subunits of Etx or
              Ctx; or

(ii) an agent having an effect on GM-1 mediated
              intracellular signalling events, but no GM-1
20   binding activity.

16.   A method for the vaccination of a mammalian subject in which an effective amount of an agent selected from:

(i)   an agent having GM-1 binding activity other
25   than Ctx and Etx; or

(ii) an agent having an effect on GM-1 mediated
              intracellular signalling events, but no GM-1
              binding activity;

is co-administered to a mammalian subject with an
30   effective amount of an unrelated foreign antigenic determinant.
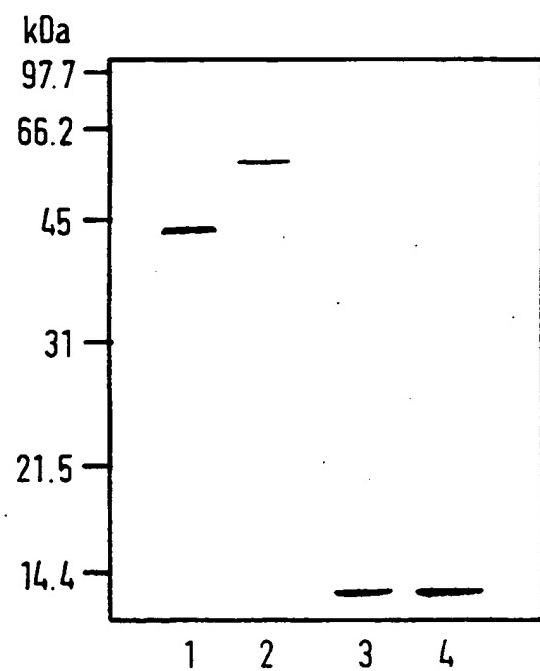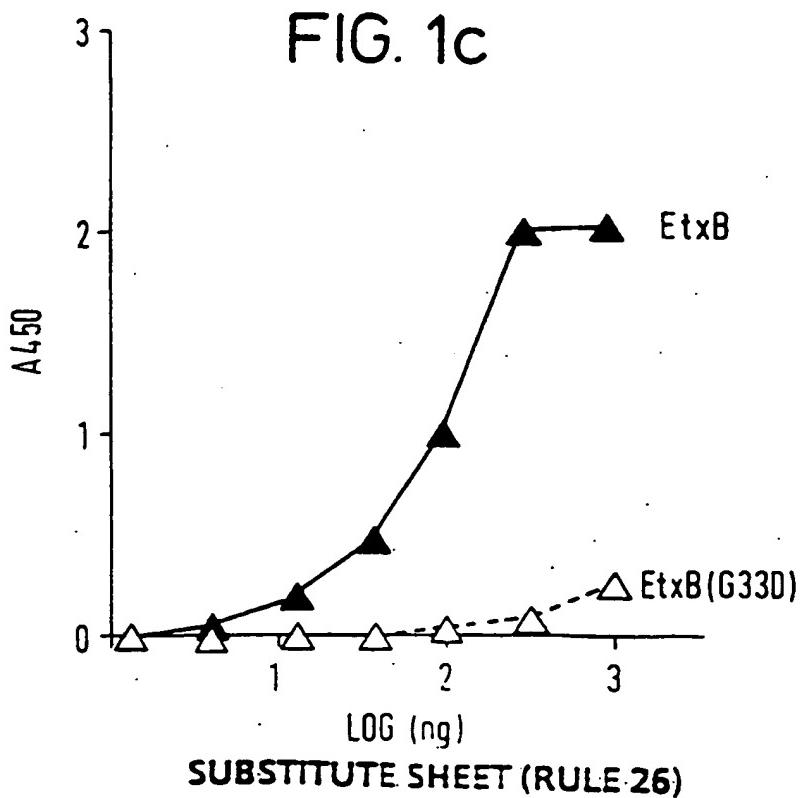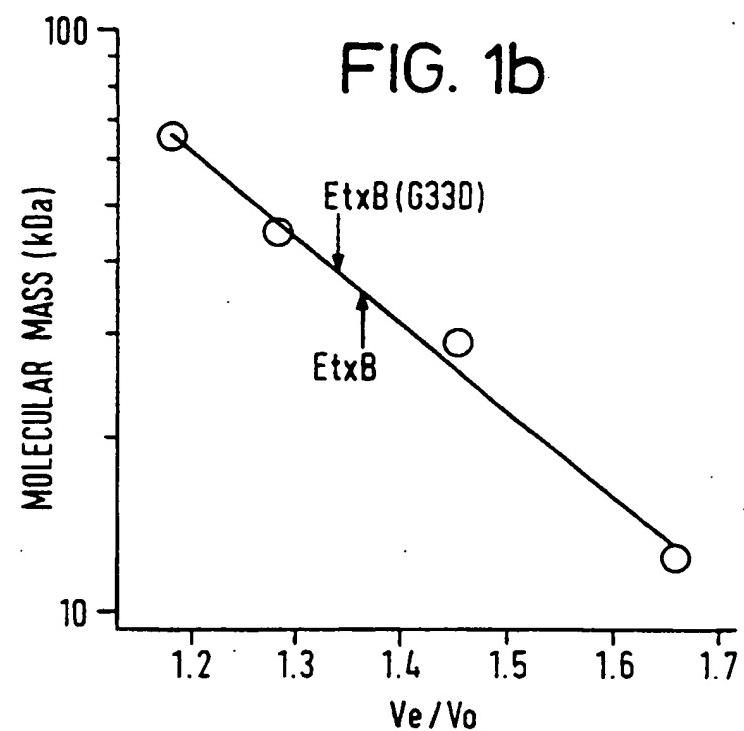
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FIG. 1a

FIG. 1b

FIG. 1c

FIG. 2

FIG. 3

FIG. 4

FIG. 5

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FIG. 6

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FIG. 7

FIG. 8

LINEAR RED FLUORESCENCE
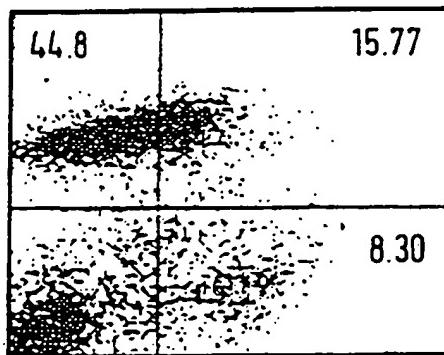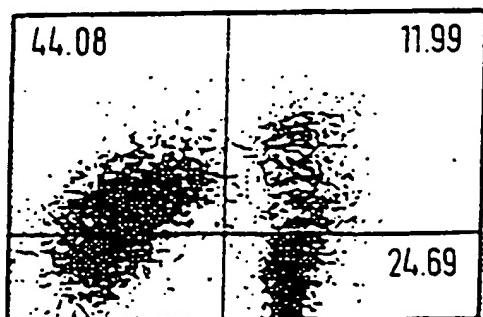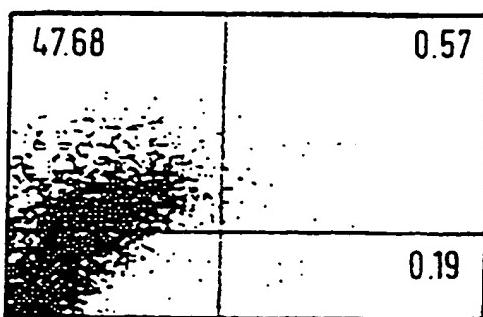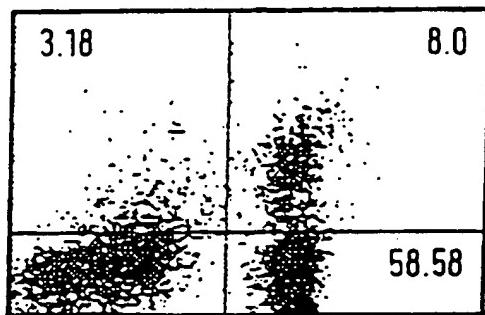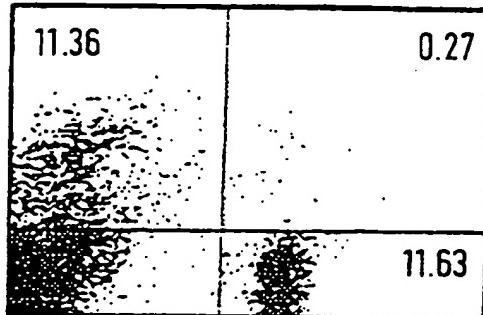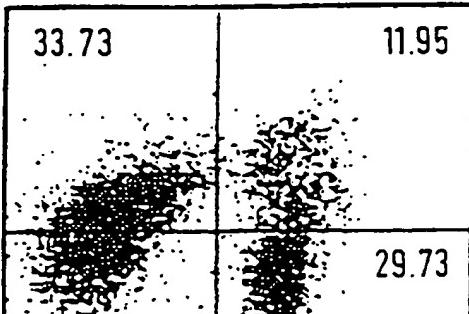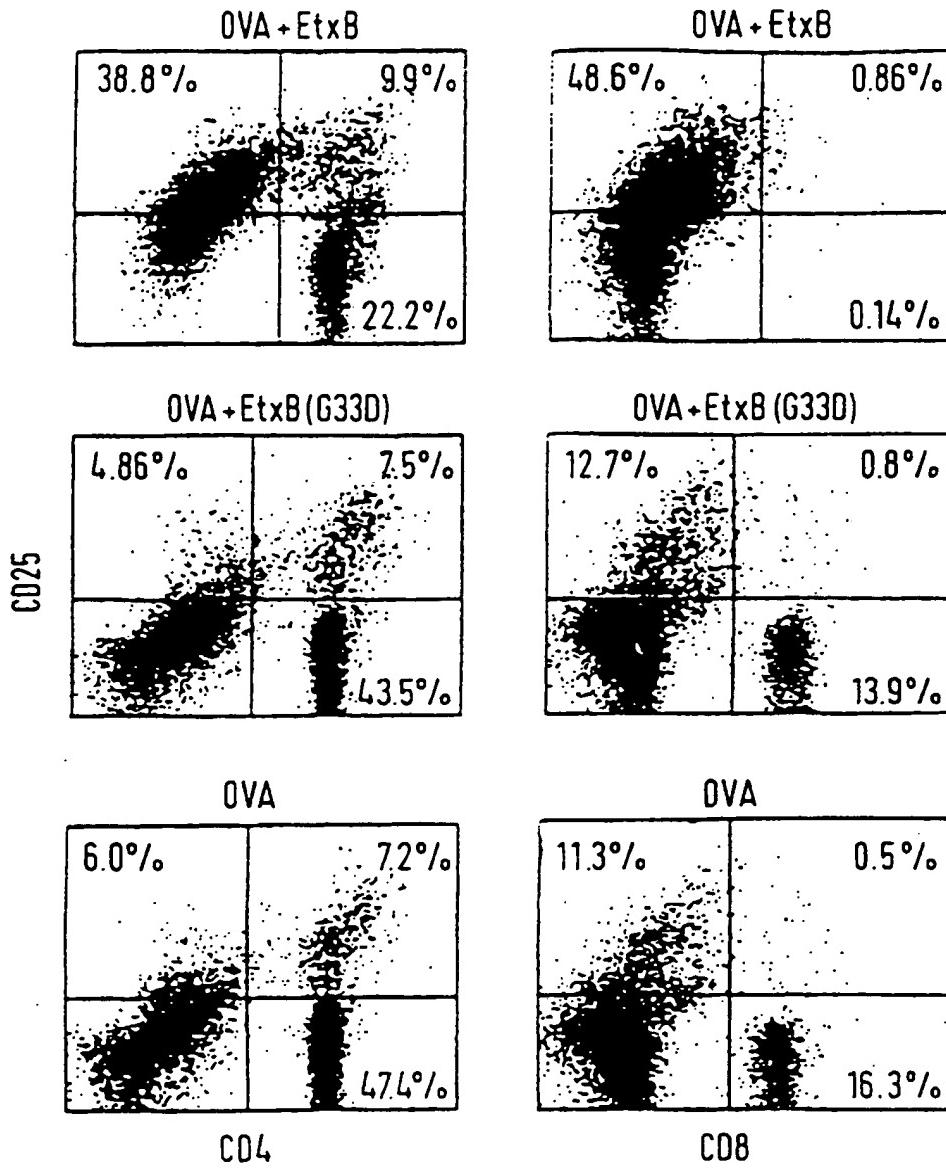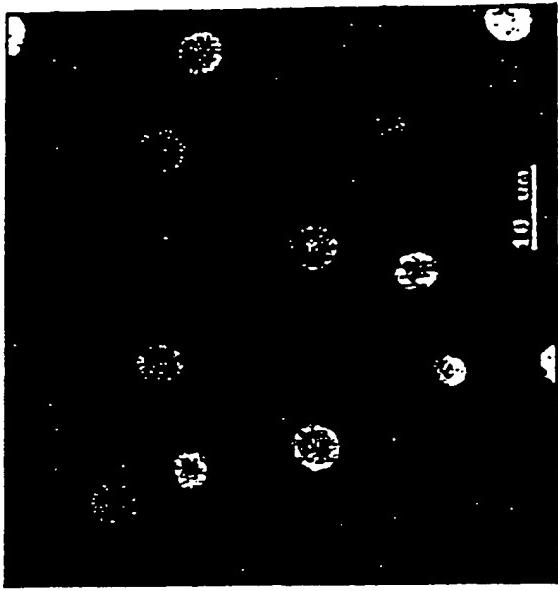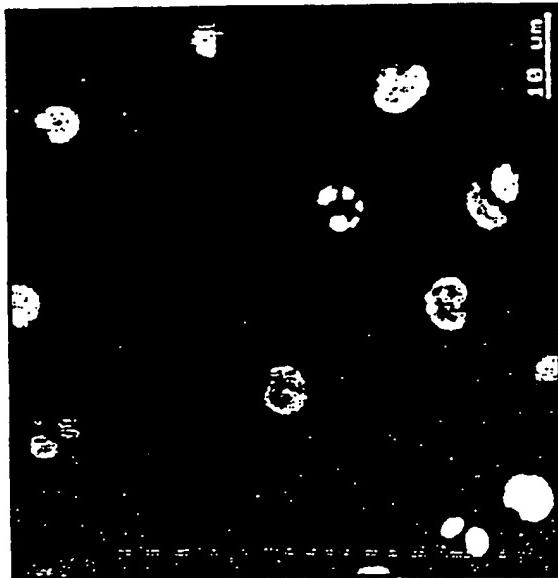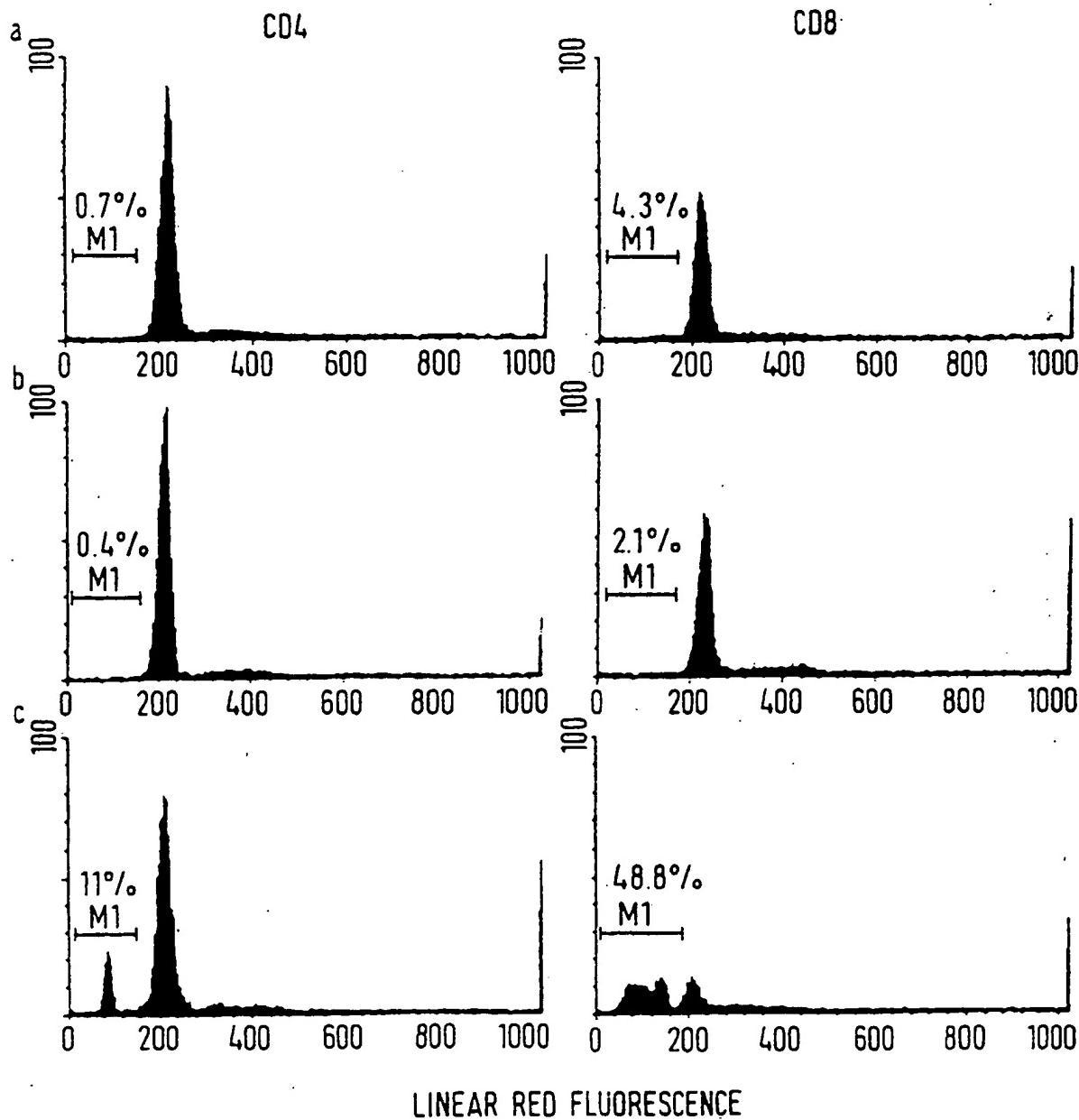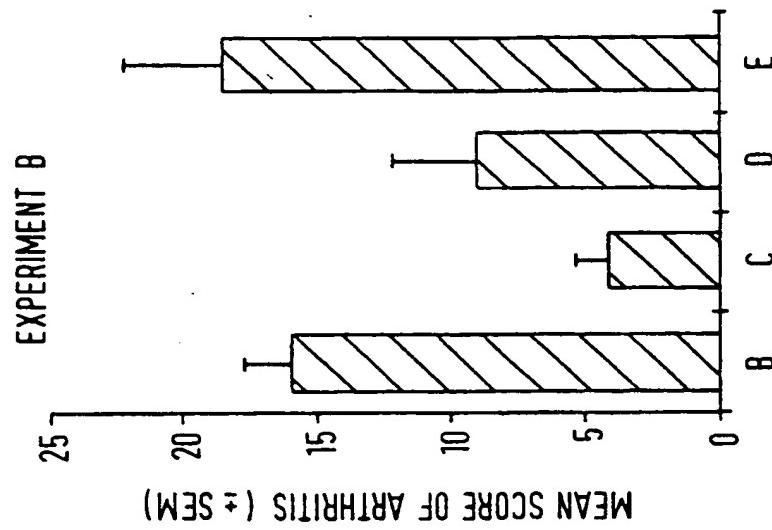
FIG. 9b
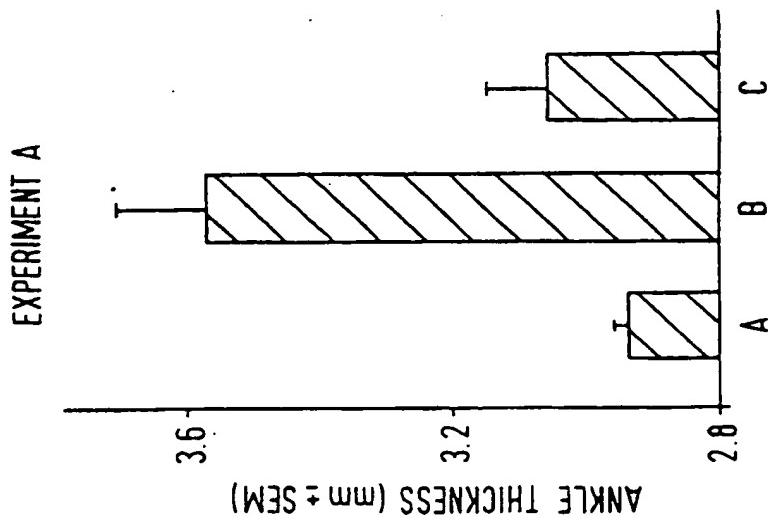
FIG. 9a

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FIG. 10
## GM1-MEDIATED APOPTOSIS OF HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS
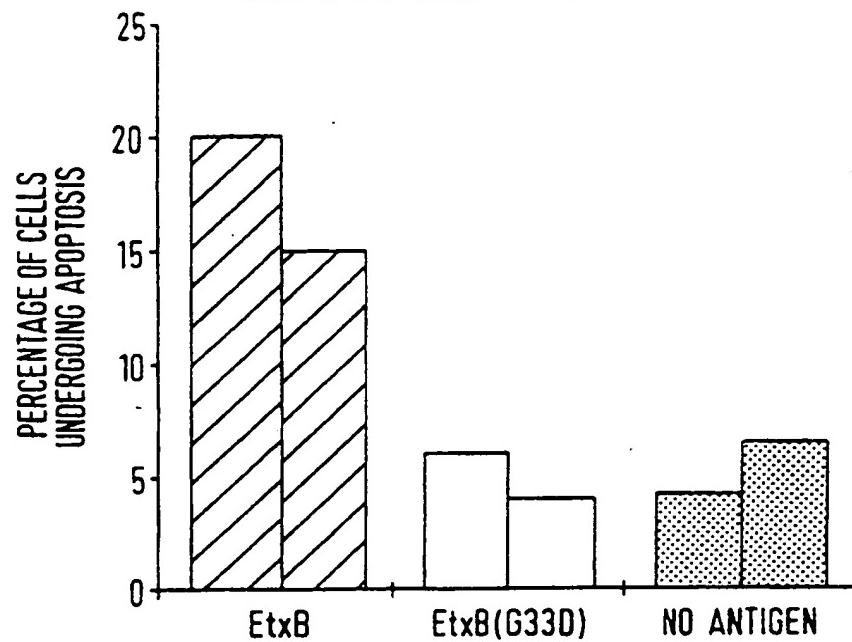
FIG. 11
GM1-MEDIATED APOPTOSIS OF AN IMMORTALISED MURINE T-CELL LINE, CTLL
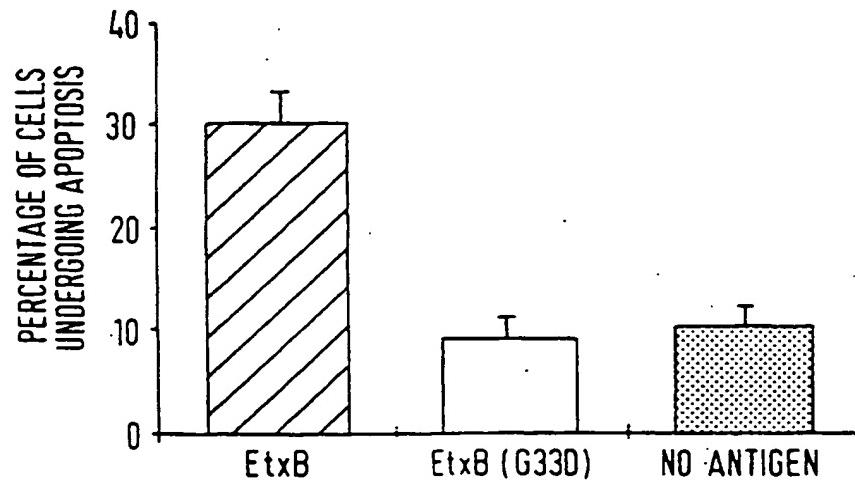
SUBSTITUTE SHEET (RULE 26)

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6   A61K39/00   C07K16/18

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6   A61K   C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA 93 (1). 1996. 226-230. ISSN: 0027-8424, XP002019422 NASHAR T O ET AL: "Potent immunogenicity of the B subunits of Escherichia coli heat-labile enterotoxin: Receptor binding is essential and induces differential modulation of lymphocyte subsets."	1-16
Y	see pages 229-230, paragraph "Discussion"	1-16

-/--

[X] Further documents are listed in the continuation of box C.   [X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 November 1996	17. 12. 96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Halle, F

Form PCT/ISA/210 (second sheet) (July 1992)

	Internati. Application No
	PCT/GB 96/01614

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	INTERNATIONAL IMMUNOLOGY 8 (5). 1996. 731-736. ISSN: 0953-8178, XP002019423 NASHAR T O ET AL: "Cross-linking of cell surface ganglioside GM1 induces the selective apoptosis of mature CD8+ T lymphocytes." see page 733, right-hand column, last paragraph - page 735, right-hand column, last line	1-16

Y	THE JOURNAL OF IMMUNOLOGY, vol. 154, 1995, pages 3611-3617, XP002019424 B. YANKELEVICH ET AL.: "Prevention of Acute Graft-Versus-Host Disease" see pages 3615-3617, paragraph "Discussion"	1-16

Y	WO,A,95 10301 (HOLMGREN JAN ;CZERKINSKY CECIL (SE)) 20 April 1995 see page 9, line 7 - page 9, line 16; claim 5	1-16

Box I   Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

   Remark: Although claim(s) 3, 4, 10, 14, 16
          is(are) directed to a method of treatment of the human/animal
          body, the search has been carried out and based on the alleged
          effects of the compound/composition.

2. [ ] Claims Nos.:
   because they relate to parts of the International Application that do not comply with the prescribed requirements to such
   an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II   Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all
   searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment
   of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report
   covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is
   restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1992)

				International Application No
				PCT/GB 96/01614

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9510301	20-04-95	AU-A-	7867194	04-05-95
		CA-A-	2173254	20-04-95
		EP-A-	0722340	24-07-96
		SE-A-	9303301	09-04-95

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